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{Exhibit 45}

Weissback, A., and Poonian, M., "Nucleic Acids Attached to Solid Matrices," Methods in Enzymology, Vol. XXXIV, Part B: 463-475 (1974)

[54] Nucleic Acids Attached to Solid Matrices

By ARTHUR WEISSBACH and MOHINDAR POONIAN

The attachment of DNA and RNA to insoluble matrices for subsequent purification of proteins or nucleic acids is a technique that has received wide use in molecular biology. Gilham[1] and Erhan *et al.*[2] have previously reported the attachment of polynucleotides to cellulose in either nonaqueous or aqueous solvents using carbodiimide reagents. Gilham has also described the binding of tRNA to aminoethyl-cellulose after periodate oxidation.[1] The binding of DNA to cellulose by adsorption has been reported by Litman[3] and well discussed by Alberts and Herrick.[4] Since these earlier reports, the binding or attachment of nucleic acids and polynucleotides to agarose, acrylic gels, and fiberglass has been accomplished. The versatility, capacity, and ease of preparation of polynucleotide matrices has established affinity chromatography as a common laboratory technique in the study of nucleic acids and enzymes.

In the following discussion, our objective is 2-fold: (a) to give detailed experimental procedures for preparing these nucleic acid–solid matrix substrates; (b) to describe some recent applications of the nucleic acid–solid matrix preparations.

Preparation of Polynucleotide Matrices

Covalent Attachment of DNA to Sephadex G-200

In principle this is an adaptation of Gilham's[1] method of condensing the 5′-phosphate end of the nucleotide with hydroxyl functions of the solid substrates. In the presence of a water-soluble carbodiimide, DNA was chemically attached to Sephadex G-200. The procedure used in the attachment is described.

Attachment of Rat Liver DNA to Sephadex.[5] Sephadex G-200, after swelling overnight in distilled water, is washed on a Büchner funnel with 6 volumes of 1 *M* NaCl, 10 m*M* Tris chloride (pH 7.4). It is further washed with 10 volumes of distilled water and finally with 2 volumes

[1] P. T. Gilham, this series, Vol. 21, p. 191.
[2] S. Erhan, L. G. Northrup, and F. R. Leach, *Proc. Nat. Acad. Sci. U.S.* 53, 646 (1965).
[3] R. Litman, *J. Biol. Chem.* 243, 6222 (1968).
[4] B. Alberts and G. Herrick, this series, Vol. 21, p. 198.
[5] D. Rickwood, *Biochim. Biophys. Acta* 269, 47 (1972).

METHODS ENZYMOL., 1974, 34(AFFINITY TECH.: ENZYME PURIF., PART B) 463-75.

of ethanol before being air dried in an oven at 60°. The dried Sephadex is ground to a fine powder before use.

1-Cyclohexyl-3-(-2-morpholinethyl) carbodiimide metho-*p*-toluenesulfonate, 250 mg, is dissolved in 3.75 ml of 0.04 *M* sodium 2-(*N*-morpholinoethane sulfonate (pH 6.0) containing 30 mg of sonicated DNA [Size range 6 to 7 S]. The reaction mixture is then spread evenly over the bottom of a glass dish (170 cm^2) and 250 mg of dry G-200 is sprinkled evenly over the bottom of the dish.[5a] The dish containing the DNA-Sephadex mixture is placed in an oven at 42° for 8 hours and transferred to a water saturated atmosphere at 20° for 24 hours.

The DNA-Sephadex is suspended in 0.15 *M* NaCl, 15 m*M* sodium citrate, and 10 m*M* Tris chloride (pH 7.4). After 4 hours the swollen Sephadex is transferred to a Büchner funnel and washed with 6 volumes of distilled water, 20 volumes of 1 *M* NaCl–10 m*M* Tris chloride (pH 7.4), and finally with 6 volumes of 0.15 *M* NaCl–15 m*M* sodium citrate containing 10 m*M* Tris chloride (pH 7.4). It may be stored as a slurry at 0° in this medium with $CHCl_3$ as a preservative.

The amount of DNA bound to the Sephadex can be determined as follows: The gel is equilibrated with distilled water, washed twice with 10 ml of ethanol, and dried at 80°. If this residue is digested in 2 ml of 0.4 *M* $HClO_4$ at 90° for 20 minutes, the DNA present in the hot acid extract can be determined by the diphenylamine method.[6]

As shown by the results presented in Table I, sonication of DNA is essential to avoid nonspecific and noncovalent binding. Under optimum conditions, 38 mg of DNA were bound per gram of Sephadex G-200. In the case of Sephadex G-25, binding is very inefficient.

The DNA bound to Sephadex is susceptible to the hydrolytic action of pancreatic deoxyribonuclease. In the presence of RNA polymerase and all four nucleoside triphosphates, the Sephadex-bound DNA could direct the synthesis of RNA. However, the template capacity of the bound DNA was only one-tenth that of an equivalent amount of free DNA.

Attachment of Nucleic Acids to Agarose

Sepharose matrices, a beaded form of agarose, offer the advantage of high capacity and good flow rates. The covalent binding of nucleic acids to Sepharose was first reported by Poonian, Schlabach, and Weissbach[7] and is described here.

[5a] D. Rickwood (personal communication).
[6] W. C. Schneider, this series, Vol. 21, p. 680 (1957).
[7] M. S. Poonian, A. J. Schlabach, and A. Weissbach, *Biochemistry* 10, 420 (1971).

TABLE I
BINDING OF DNA TO SEPHADEX[a]

DNA[b]	Incubation conditions	Milligrams of DNA bound per gram of Sephadex	Binding (%)
Highly polymerized	Standard	15	50
	Omit carbodiimide	13	43
Sonicated	Standard	9	30
	Omit carbodiimide	0	0
	Dry at 20°	6	20
	75 mg of carbodiimide per reaction mix	8	27
	100 mg of Sephadex G-200 per reaction mix	10	34
	6.0 mg of DNA per reaction mix	38	32
	Replace Sephadex G-200 by Sephadex 25	0.5	1

[a] From D. Rickwood, *Biochim. Biophys. Acta* **269**, 47 (1972).

[b] DNA, highly polymerized (greater than 30 S) or sonicated (6–7 S), was incubated under the conditions shown. The DNA remaining bound to the Sephadex after washing was digested in 0.4 *M* $HClO_4$ at 90°, and the solubilized DNA was estimated as described in the text. All values are the mean of two separate incubations using different batches of DNA and Sephadex.

Procedure for Substrate Preparation. The method was patterned after that of Cuatrecasas[8] in which a ligand with an amino group was attached to Sepharose by forming a putative chemical link between the hydroxyl of Sepharose and nitrogen of the amino group. In preparing the nucleic acid–Sepharose the following procedure is used. A slurry of a known volume of Sepharose 4B is obtained by mixing it with an equal volume of water. The slurry container is surrounded with a jacket of iced water to maintain the temperature below 10° during the reaction. The pH of the reaction is determined by keeping the electrode of the pH meter in the reaction mixture throughout the reaction. A calculated amount of well powdered cyanogen bromide (200–500 mg/ml of Sepharose) is added in one lot to the gently stirred slurry and dropwise addition of 2–5 *M* sodium hydroxide is started immediately so as to maintain the pH at 11. Cessation of the decrease in pH below 11 and simultaneous disappearance of solid cyanogen bromide indicates completion of the activation reaction. The slurry of activated Sepharose is immediately cooled by adding ice to it and is then filtered and washed three times with 10 volumes of 50 m*M* potassium phosphate (pH 8.0) under suction without allowing the activated Sepharose to dry. The activated complex

[8] P. Cuatrecasas, *J. Biol. Chem.* **245**, 3059 (1970).

is added to the nucleic acid ligand in a volume of 50 m*M* potassium phosphate at pH 8.0 equal to that of packed Sepharose used in the reaction; the mixture is stirred gently at 4° for a period of 16–48 hours. The reaction slurry is packed into an appropriate column and washed with 50 m*M* potassium phosphate (pH 8.0) until nucleic acid no longer appears in the wash as determined by radioactivity or by $A_{260\ nm}$. To ensure complete removal of noncovalently attached material, a final wash of 0.5 *M* potassium phosphate at pH 8.0 is used. Quantitation of the conjugation may be based upon the difference between input and wash radioactive counts and/or A_{260}. In case of radioisotope-labeled material, direct counts were also taken on coupled Sepharose by making a slurry in appropriate buffer and counting an aliquot.

The results of coupling various DNA species to activated Sepharose are presented in Table II. With the exception of poly[d(A-T)], the other DNA species measured do not undergo a significant coupling reaction when present in a native double-stranded form. In contrast, extensive binding occurs when a double-stranded species is denatured to the single-stranded form. Thus 20% of denatured *E. coli* DNA can be attached to agarose whereas none of the original native DNA binds under these conditions. Similarly, the introduction of single-stranded ends into a double-stranded DNA permits attachment of the nucleic acid to occur. As shown in Table II, native λ DNA, a double-stranded structure containing complementary single-stranded 5′ ends 12 nucleotides in length[9] does not bind to Sepharose. If 5′ ends of the λ DNA are degraded 12.5% on each side (about 6000 nucleotides) with the λ exonuclease, 40% of the DNA can be attached. It is important to note that the digestion of double-stranded DNA by the λ-exonuclease to yield single-stranded ends must be carried out at high pH (9.5), where the enzyme acts in a random fashion. At lower pH values, the enzyme processively and preferentially digests the molecule to which it initially binds.[10] The dependency of binding on single strandedness is further illustrated by the results with HeLa DNA wherein the percentage of DNA which can be attached to Sepharose increases as the amount of single-stranded ends increases. However, the amount of DNA which the Sepharose can bind is apparently limited since at a high DNA:Sepharose ratio (10 A units per milliliter of Sepharose) the efficiency of attachment seems to decrease. In case of poly[d(A-T)] the pH of the solution (pH 8.0) is probably enough to "soften" the double-stranded hydrogen bonding and allow the coupling to proceed.

[9] R. Wu and A. D. Kaiser, *J. Mol. Biol.* **35**, 523 (1968).
[10] D. M. Carter and C. M. Radding, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **29**, 896 (1970).

TABLE II
ATTACHMENT OF NUCLEIC ACIDS TO SEPHAROSE[a]

Nucleic acid	Packed Sepharose volume (ml)	CNBr (g)	Nucleic acid used		Amount coupled to Sepharose		
			A_{260}	Cpm	$A_{260/ml}$	Total A_{260}	%
[^{14}C]Poly(dA-T)	5	1	0.91	2.2×10^5	0.136	0.68	75
^{3}H *Escherichia coli* DNA (native)	5	1.3	29.6	1.5×10^7	0	0	0
^{3}H *E. coli* DNA (native) (sonicated)	5	1.2	8.7	5×10^5	0	0	0
^{3}H *E. coli* DNA (native) (coupling performed at pH 10.65)[b]	5	1.3	4.3	4×10^5	0.017	0.085	2
^{3}H *E. coli* DNA (denatured)	5	1.3	11.05	4×10^5	0.461	2.3	20.9
^{3}H λ DNA (native)	5	2.26	6.0	1×10^5	0		0
^{3}H λ DNA (12.5% single-stranded end on each side)	10	5.0	29.8	5×10^5	1.19	11.9	40
^{3}H HeLa DNA (native)							
2.2% single-stranded end on each side	5	1.8	5.0	5×10^5	0.043	0.215	4.3
3.8% single-stranded end on each side	10	4.95	39.0	5×10^5	0.663	6.63	17
8.3% single-stranded end on each side	5	1.5	2.0	2×10^5	0.192	0.96	24
^{3}H HeLa DNA (denatured)	30	9.0	300	1×10^6	0.90	27	9.0
Polyadenylic acid	10	2	77.0		7.5	75	98

[a] All coupling reactions were carried out in 50 m*M* sodium or potassium phosphate buffer (pH 8.0) unless otherwise indicated.
[b] Sodium carbonate buffer, 50 m*M* was used.

With Sepharose-bound DNA it has been possible to partially purify HeLa cell DNA polymerase[7] and both bovine pancreatic and hog spleen deoxyribonuclease.[11]

Binding of RNA to Sepharose

CNBr-activated Sepharose can also be used as a matrix for the attachment of single-stranded RNA or synthetic polynucleotides. Wagner *et al.*[12] have coupled RNA and poly(I:C) to Sepharose and the attachment of poly(U), using this technique, has been done by Lindberg and Persson.[13] Although binding of poly(A) to Sepharose at pH 8.0 has been reported[7] (Table II), more efficient attachment (>90%) of polynucleotides to CNBr activated Sepharose is more consistently obtained at pH 6.0 under the conditions of Wagner *et al.*,[12] as described below.

Sepharose 4B is washed with 0.1 *N* NaCl and then with deionized water. For the activation reaction, 15 ml of washed Sepharose 4B are suspended in 30 ml of deionized water and cooled to about 10°. After the addition of 4.5 g of CNBr to the stirred suspension, the pH is maintained at 11 by the addition of 8 *N* NaOH. The temperature rises to 20° in 15 minutes. The activated matrix material is placed on a filter and washed with 600 ml of cold deionized water. It can be used promptly in the condensation reaction or after several weeks of storage in deionized water at 4°.

The binding of single-stranded RNA to Sepharose is accomplished by adding 3 volumes of a solution (3–4 mg/ml) of the ribonucleic acid in 0.2 *M* 4-morpholinoethanesulfonic acid buffer (pH 6.0) to 1 volume of activated Sepharose. The suspension is stirred overnight at 4°, and the product is isolated on a filter. The Sepharose-bound ribonucleic acid is packed in a column and washed continuously with 700 ml of 0.15 ml NaCl–6 m*M* sodium phosphate at pH 7.0. The amount of ribonucleic acid binding to activated Sepharose is established by hydrolysis of an aliquot of the product in 10 volumes of 0.25 *N* NaOH overnight and measurement of the ultraviolet absorption of the filtrate at pH 7.0.

Attachment of RNA to Agarose with DDC

The attachment of RNA by its 3′ terminus to agarose has been accomplished by Robberson and Davidson.[14] Their procedure used alkaline activation of agarose with CNBr followed by coupling of ε-aminocaproic

[11] J. C. Schabort, *J. Chromatogr.* 73, 253 (1972).
[12] A. F. Wagner, R. L. Bugianesi, and T. Y. Shen, *Biochem. Biophys. Res. Commun.* 45, 184 (1971).
[13] U. Lindberg and T. Persson, *Eur. J. Biochem.* 31, 246 (1972).
[14] D. L. Robberson and N. Davidson, *Biochemistry* 11, 533 (1972).

acid methyl ester to the agarose. The caprolyl ester-agarose is then converted to the corresponding hydrazide. After further blockage of resin carboxyl groups with glycinamide and water soluble carbodiimide, the treated agarose is coupled with RNA which had previously been oxidized with sodium periodate. Although rather more complicated, this procedure may have specialized use.

Entrapment of DNA in Agarose

These matrices are prepared according to the procedure of Bendich and Bolton[15] for immobilizing DNA in agar. In contrast with the covalently attached DNA-matrices discussed above, the mode of attachment here involves entrapment of single-stranded DNA molecules in the agarose matrix. DNA-agarose preparations containing 3.5 mg of calf thymus DNA per milliliter of bed volume or 0.75 mg of FD-circular DNA per milliliter of agarose have been described by Schaller *et al.*[16] They have shown that 6% of the soluble *E. coli* proteins are retained on the DNA agarose and can be eluted with increasing salt concentrations. The enzymes purified with columns of this type include *E. coli* DNA polymerases I and II, exonuclease III, RNA polymerase, T4 polynucleotide kinase[16] and the *E. coli* ribonucleases.[17]

Nucleic Acid–Cellulose Matrices

These are the most commonly used materials in nucleic acid affinity chromatography. The attachment of polynucleotides to cellulose with carbodiimide reagents has been reviewed by Gilham[1] and has been successfully used in many laboratories. DNA has also been bound to cellulose by adsorption techniques,[4] and Litman[3] has used ultraviolet light irradiation to bind DNA to cellulose. The Litman technique as described below, is very efficient and leads to high DNA ratios of cellulose.

Preparation of DNA-Cellulose.[3] Cellulose, 15 g (Solka-Floc, Brown Company, Berlin, New Hampshire), is washed by stirring for 10 minutes in 300 ml of 1 *N* HCl, filtered on Whatman No. 1 paper in a Büchner funnel, and rinsed thereon with water. The cellulose is then stirred with another 200 ml of 1 *N* HCl for 10 minutes, again filtered and washed exhaustively with water until no trace of acid remains, and finally spread out to dry in air.

DNA, 16 mg in 8 ml of 1 or 10 m*M*, NaCl, is mixed with 1 g of

[15] A. J. Bendich and E. T. Bolton, this series, Vol. 12B, p. 635.
[16] H. Schaller, C. Nüsslein, F. J. Bonhoeffer, C. Kurz, and I. Nietzschmann, *Eur. J. Biochem.* 26, 474 (1972).
[17] S. C. Weatherford, L. S. Weisberg, D. T. Achord, and D. Apirion, *Biochem. Biophys. Res. Commun.* 49, 1307 (1972).

acid-washed cellulose. Adequate mixing is achieved by kneading with a spatula; the paste is spread thinly over the surface of 250-ml beaker and dried with cool air for several hours. The preparation is allowed to remain at room temperature overnight and is then scraped from the sides of the beaker and suspended in absolute alcohol. A low pressure mercury lamp (Mineralight, Ultra-Violet Products, Inc., South Pasadena, California) is placed at a distance of about 10 cm from the surface of the alcohol suspension and irradiation (about 100,000 ergs/mm^2), is carried out for 15 minutes with continuous slow stirring. The slurry is filtered with suction on Whatman No. 2 paper, and the filter cake is washed three times by stirring in 50 ml of 1 m*M* NaCl for 10 minutes. After filtration, the preparation is spread out on filter paper to dry in air. About 10% of the DNA was found to be bound to the cellulose in the absence of irradiation and 90% after irradiation.[3] Preparations may be stored at room temperature for several years.

RNA-Cellulose

This technique using ultraviolet light irradiation has been extended by Smith and co-workers[18] to the attachment of RNA to methylated cellulose as follows: 5 g of methylated cellulose are added to 8–15 mg of RNA in 20 ml of 5 m*M* NaCl, and the mixture is dried overnight under a stream of air. Methylation is used to block the cellulose carboxyl groups and minimizes nonspecific adsorption of nucleic acids. The dried RNA-cellulose is resuspended in 100 ml of absolute ethanol and irradiated for 30 minutes at 15 cm from a low-pressure mercury lamp (100,000 ergs/mm^2) with continuous stirring. After removal of ethanol by filtration, the RNA-cellulose is washed with 4–6 liters of 5 m*M* NaCl. About 30% of the input RNA is bound to cellulose under these conditions.

Coupling of Nucleic Acids to Other Insoluble Matrices

The use of ultraviolet light irradiation as a "binder" has also been extended to other matrices. Sheldon *et al.*[19] have attached both polyuridylic and polycytidylic acid to fiberglass filters in the presence of ultraviolet light and used these filters for isolation of messenger RNA.

Preparation and Use of Poly(U) and Poly(C) Filters.[19] One hundred and fifty microliters of poly(U) solution (1 mg/ml in water) are added to a fiberglass filter (Whatman GF/C, 2.4 cm in diameter). The filter is dried at 37° and irradiated for 2.5 minutes on each side at a distance

[18] I. Smith, H. Smith, and S. Pifko, *Anal. Biochem.* 48, 27 (1972).
[19] R. Sheldon, C. Jurale, and J. Kates, *Proc. Nat. Acad. Sci. U.S.* 69, 417 (1972).

of 22 cm from a 30 W Sylvania germicidal lamp. Each filter is washed with 50 ml of distilled water to remove unbound poly(U). About 65% of the poly(U) applied is retained on the filter; ten times more poly(U) is retained if the input is increased 10-fold.

The same technique can be applied to the preparation of poly(C) filters except that the filters are equilibrated with 0.45 *M* NaCl–45 m*M* sodium citrate, and this buffer is used for the application of sample and washing of the filters. The remainder of the procedure is as described above.

Radioactive RNA to be tested for binding to poly(U) may be dissolved in any convenient amount (25 μl to 10 ml) of the binding buffer (10 m*M* Tris chloride of pH 7.5 containing 0.12 *M* NaCl). The sample is applied either without suction for very small volumes or at a suction rate of 2 ml per minute for larger volumes. The filters are washed first with water and then with buffer before the sample is applied.

The sample is applied at room temperature. Once the sample is applied, one waits a minute or two and then washes the filters sequentially with the binding buffer, with 5% TCA and with 95% ethanol. The filters are dried and counted.

If it is desired to re-use these fiberglass filters, the TCA and ethanol washes are omitted. The filters are washed instead with 0.30 *M* NaCl-0.03 *M* sodium citrate, dried and then counted in a toluene based scintillation fluid (Liquifluor). For regeneration, the filters are removed from the scintillation fluid and washed with pure toluene. After drying, they are suspended in 15 m*M* NaCl–1.5 m*M* sodium citrate (10 ml per filter) at 65° for 1 hour, and each filter is subsequently washed with 20 ml of this buffer at 65°. This procedure elutes the hybridized RNA, leaving behind the original poly(U)-fiberglass filter.[20]

Matrices of Acrylic Polymers in Agar. The coupling of periodate oxidized RNA to a linear polymer of acrylic hydrazide entrapped in agar has been reported by Petre *et al.*[21] and is based on a procedure first described by Knorre *et al.*[22] These acrylic matrices have been bound to messenger-RNA,[21] tRNA,[23] and polyuridylic acid,[24] and subsequently used for the isolation of ribosomes from *E. coli.*

[20] A. Skalka, unpublished observations.
[21] J. Petre, A. Bollen, P. Nokin, and H. Grosjean, *Biochim.* 54, 823 (1972).
[22] D. G. Knorre, S. D. Misina, and L. C. Sandachtchiev, *Izv. Sib. Otd. Akad. Nauk SSSR Seriya Khim. Nauk* 11, 134 (1964).
[23] O. D. Nelidova and L. L. Kiselev, *Mol. Biol. (USSR)* 2, 47 (1968).
[24] L. L. Kiselev and T. A. Ardonina, *Mol. Biol. (USSR)* 3, 88 (1971).

Applications of Polynucleotide Matrices

Polynucleotide-cellulose combinations provide an excellent measure of the versatility of nucleic acid matrices. The following examples show the use of polynucleotide-cellulose for the isolation of messenger RNA, the purification of an RNA dependent DNA polymerase, and as an actual substrate for the enzyme polynucleotide ligase.

Purification of Biologically Active Globin Messenger RNA by Chromatography on Oligothymidylic Acid-Cellulose[25]

A convenient technique for partial purification of large quantities of functional polyadenylic acid-rich mRNA has been used for the isolation of rabbit globin mRNA. Since many mammalian and viral mRNA's appear to be rich in polyadenylic acid, this approach should prove to be generally useful as an initial step in the isolation of such mRNA's. Oligothymidylic acid–cellulose columns are prepared according to the procedure of Gilham,[26] and RNA is chromatographed on these columns as follows:

All operations are performed at room temperature with sterile glassware and reagents [except for oligo(dT)-cellulose]. Crude rabbit reticulocyte polysomal RNA [100 A_{260} units] is dissolved in a buffer containing 10 m*M* Tris chloride (pH 7.5)–0.5 *M* KCl and applied to a 2-ml (about 0.5 g, dry weight) oligo(dT)–cellulose column equilibrated with this buffer. The nonabsorbed material is eluted by continued washing with the same buffer. The material retained by the column is then eluted sequentially with buffers of reduced ionic strength. The first elution buffer contains 10 m*M* Tris chloride at pH 7.5–0.1 *M* KCl; the second, 10 m*M* Tris chloride at the same pH. The material eluted by either of these buffers is precipitated by the addition of CH_3COOK [final concentration of 2%] and 2 volumes of ethanol or is frozen directly and stored in liquid nitrogen. The oligo(dT)-cellulose can be regenerated for further use by washing with 0.1 *M* KOH.

Over 95% of the total polysomal RNA applied to the oligo(dT)-cellulose column is eluted with the application buffer, and the rest is retained by the column. When the KCl concentration of the elution buffer was reduced from 0.5 to 0.1 *M*, an additional small amount of UV-absorbing material is eluted. Final elution with 10 m*M* Tris chloride at pH 7.5 releases an additional peak of RNA from the oligo(dT)-cellulose. This last peak exhibits the highest protein-synthesizing activity in a cell-

[25] H. Aviv and P. Leder, *Proc. Nat. Acad. Sci. U.S.* 69, 1408 (1972).
[26] P. Gilham, *J. Amer. Chem. Soc.* 86, 4982 (1964).

free protein-synthesizing system, and the major activity in the peak was shown to sediment in sucrose gradients at approximately 9 S.

Purification of RNA-Dependent DNA Polymerase from RNA Tumor Viruses[27]

The polymerase from Rauscher murine leukemia virus has been purified by a one-step enrichment procedure on columns of $(dT)_{12-18}$-cellulose. Advantage is taken of the property of viral polymerases to preferentially bind to $(dT)_{12-18}$ primers.[28]

Procedure. An aqueous suspension of virus is diluted with an equal volume of buffer containing 0.1 *M* Tris chloride (pH 7.8), 1.0 *M* KCl, 2 m*M* dithiothreitol, 2% Triton X-100, 40% (v/v) glycerol. The suspension is incubated for 30 minutes at 37° and centrifuged at 100,000 *g* for 1 hour at 4°. The supernatant fluid is dialyzed for 1 hour against 250 volumes of buffer containing 10 m*M* potassium phosphate (pH 7.1), 1 m*M* dithiothreitol, 60 m*M* KCl, 0.1% Triton X-100, 20% (v/v) glycerol, and 0.5 m*M* manganese acetate. The sample is applied to a $(dT)_{12-18}$–cellulose[26] column, equilibrated with the same buffer, and eluted with a linear KCl gradient in this buffer containing no Mn^{2+}. The RNA-dependent DNA polymerase elutes as a sharp peak at approximately 0.25 *M* KCl. The columns may be washed with 1 *M* KCl and reused, but this should not be done more than four times.

After the $(dT)_{12-18}$–cellulose column enrichment, the enzyme can be further purified by phosphocellulose chromatography and gel filtration on Sephadex G-100.

Polynucleotide-Cellulose as a Substrate for a Polynucleotide Ligase Induced by Phage T4[29]

In this procedure polynucleotide-cellulose is used as substrate in assaying a T4-induced polynucleotide ligase. The assay is rapid and sensitive and directly measures the linkage of two polynucleotide strands. The starting material is the interrupted homopolymer pair of dI:dC schematically depicted in Fig. 1.

One strand of poly(dC) is ^{3}H-labeled and the other is covalently joined to a cellulose particle. Alkaline denaturation removes the labeled poly(dC) from the cellulose (Fig. 1b) unless the interruption has been enzymatically repaired (Fig. 1c, d). Labeled strands covalently attached

[27] B. L. Gerwin and J. B. Milstein, *Proc. Nat. Acad. Sci. U.S.* 69, 2599 (1972).
[28] D. M. L. Livingston, E. M. Scolnick, W. P. Parks, and G. J. Todaro, *Proc. Nat. Acad. Sci. U.S.* 69, 393 (1972).
[29] N. R. Cozzarelli, N. E. Melechen, T. M. Jovin, and A. Kornberg, *Biochem. Biophys. Res. Commun.* 28, 578 (1967).

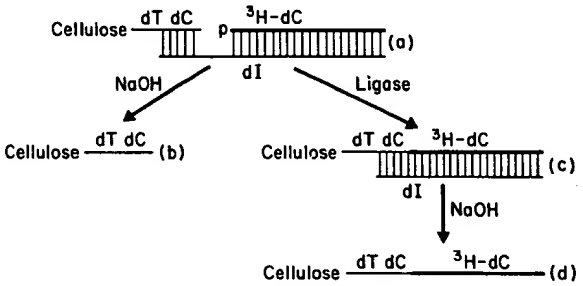

FIG. 1. Schematic representation of the assay for polynucleotide ligase as described by N. R. Cozzarelli, N. E. Melechen, T. M. Jovin, and A. Kornberg [*Biochem. Biophys. Res. Commun.* **28**, 578 (1967)]. The substrate (a) consists of dT, an oligomer of 5–10 deoxythymidylate residues esterified through its 5′-phosphoryl terminus to one of the glucose hydroxyl groups of the cellulose. The dT is in turn joined by phosphodiester linkage to dC, a polymer of 100–200 deoxycytidylate residues with a free 3′-hydroxyl group at its terminus. The dC and ^{3}H-labeled dC, a strand of about 1800 deoxycytidylate residues with free 5′-phosphoryl and 3′-hydroxyl termini, are hydrogen bonded to dI, a strand of about 1800 deoxyinosinate residues. Alkaline denaturation of (a) leads to (b), in which ^{3}H-labeled dC and dI are removed from the cellulose complex. Ligase action joins ^{3}H-labeled dC covalently to cellulose-dTdC to produce (c), alkaline denaturation of which leads to (d), in which ^{3}H-labeled dC is retained in the cellulose complex.

to cellulose are collected by filtration of the alkaline mixture and provide a measure of the number of successful strand unions.

Preparation of the Substrate. Terminal deoxynucleotidyl transferase from calf thymus[30] may be used to synthesize a poly(dC) strand attached to the oligo dT portion of oligo dT-cellulose.[20] The reaction mixture contains 20 mg of oligo dT-cellulose (about 1.5 μmoles of nucleotide residues), 200 m*M* potassium cacodylate at pH 7.0, 23 m*M* KCl, 1 m*M* $CoCl_2$, 1 m*M* β-mercaptoethanol, 1 m*M* dCTP, and 9 μg of purified terminal transferase in a total volume of 0.23 ml. After 4–8 nmoles of deoxycytidylate residues are attached per milligram of oligo dT-cellulose, the cellulose is sequentially washed with 50-ml portions of 10 m*M* EDTA, 300 m*M* NaCl, 50 m*M* NaOH, water, ethanol, and ether and dried under reduced pressure. To anneal poly(dI) to this product, 40 mg of the poly(dC)–oligo(dT)–cellulose are mixed with 1.24 ml of 1.5 m*M* EDTA, 0.19 m*M* poly(dI), and 20 m*M* NaOH to destroy any preexisting secondary structure. The mixture is adjusted to pH 7.8 with 20 m*M* sodium phosphate buffer, and made 110 m*M* with NaCl; it is stirred for 2 hours at 25° and for another 2 hours at 21°. The product is washed

[30] K. Kato, J. M. Goncalves, G. E. Houts, and F. J. Bollum, *J. Biol. Chem.* **242**, 2780 (1967).

3 times with 10 m*M* sodium phosphate at pH 7.8, containing 100 m*M* NaCl, and 0.5 m*M* EDTA. The cellulose pellet is then annealed to poly(dC) by resuspending the pellet in 0.83 ml of this buffer containing 0.11 m*M* ^{3}H-labeled poly(dC)[31] (7.6×10^7 cpm/pmole of nucleotide). The mixture is agitated for 4 hours at 21° until about 60% of the poly(dC) has been annealed. The product is washed with the sodium phosphate–NaCl–EDTA buffer and stored at 4° at a cellulose concentration of 10 mg/ml in this buffer; this is the substrate described in Fig. 1a.

The assay for polynucleotide ligase measures the covalent linking of the ^{3}H-labeled poly(dC) to the cellulose-dTdC substrate (Fig. 1).[29]

[31] M. J. Chamberlin and D. L. Patterson, *J. Mol. Biol.* **12**, 410 (1965).

[55] Immobilized Nucleotides for Affinity Chromatography

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Introduction and Principle

Nucleosides and nucleotide phosphates occur in many biological systems and act as enzyme substrates, as cofactors, and as coenzymes. Thus, covalently binding these compounds to insoluble matrices provides a useful tool for the study of their interactions with proteins[1,2] as well as media for affinity chromatography.[3,4]

A simple and general method for coupling nucleosides, nucleotide phosphate, and coenzymes possessing vicinal free hydroxyl groups to agarose hydrazide columns, has been described.[5,6] (Scheme I). These systems are prepared by reacting an excess of a dihydrazide of the structure

$$NH_2NH\text{—}\overset{O}{\overset{\|}{C}}(CH_2)_x\overset{O}{\overset{\|}{C}}\text{—}NHNH_2$$

to cyanogen bromide-activated polymer, in either sodium bicarbonate or acetic acid solutions. The coupling of periodate-oxidized nucleotides to the agarose hydrazide is carried out under mild conditions; the reac-

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[6] D. L. Robberson and N. Davidson, *Biochemistry* **11**, 533 (1972).